Enhanced cAMP Response of Naturally Occurring Mutant of Human β_3 -Adrenergic Receptor

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ABSTRACT—We have examined the functional significance of the naturally occurring mutation at position 64 of human β_3 -adrenergic receptor (β_3 AR), which changes the amino acid from tryptophan to arginine (W64R- β_3 AR). The affinities of β AR agonists for W64R- β_3 AR expressed in COS-7 cells were not significantly different from those for wild type β_3 AR. When two receptors are expressed at various expression levels, and stimulated with CGP12177A, they showed essentially the same EC_{50} values and maximal responses. Overexpression of G_i and G_o , or the treatment with pertussis toxin did not affect the agonist-induced cAMP response, suggesting that G_i and G_o did not contribute to the β_3 AR-induced cAMP response. However, the enhanced cAMP response was observed when W64R- β_3 AR was coexpressed with the adenylyl cyclase type III isoform, and stimulated by CGP12177A and isoproterenol. These results indicate that the cAMP response of W64R- β_3 AR can be enhanced under the particular condition that adenylyl cyclase type III was coexpressed.

Keywords: β_3 -Adrenergic receptor, Adenylyl cyclase isoform, Polymorphism, β -Adrenergic receptor agonist, cAMP production

The β_3 -adrenergic receptor (β_3 AR) belongs to a superfamily of G protein-coupled receptors and is mainly expressed in adipose tissue and the gastrointestinal tract (1). Stimulation of β_3 AR activates adenylyl cyclase (AC) and increases the intracellular concentration of cAMP, leading to lipolysis and thermogenesis. β_3 AR agonists are envisioned as one of the anti-obesity drugs (2).

Three groups independently reported that the mutation of tryptophan to arginine at position 64 of human β_3 AR (W64R- β_3 AR) correlates with obesity, glucose intolerance, hypertension, dyslipidemia and early onset of non-insulin-dependent diabetes mellitus (3–5). However, the functional significance of the mutation remains an unsolved issue. Candalore et al. have examined ligand binding, activation of AC and desensitization pattern, and they found that the biochemical characteristics of W64R- β_3 AR are essentially the same as those of wild type (WT)- β_3 AR (6). In contrast to their report, Pietri-Rouxel et al. reported that the degree of maximal cAMP response by W64R- β_3 AR stimulation is lower than that by WT- β_3 AR stimulation (7). They also

showed that the ligand binding characteristics of agonists and antagonists for W64R- β_3 AR and the half maximal concentrations of agonists to activate W64R- β_3 AR are same as those of WT- β_3 AR (7). There is a difference in assay system between the two groups: one group used a pool of cells that showed resistance to antibiotics, and the other group used clonal cell lines that stably expressed receptors. Thus, it remains to be determined whether W64R- β_3 AR has a decreased or increased ability to induce the cAMP response. Granneman reported that the AC type III isoform (AC-III) is selectively increased in brown adipose tissue by neural stimulation or by cold exposure (8). Therefore, it is also necessary to compare the β_3 AR-mediated responses under the condition that the expression of AC-III is increased. In the present study, we report that W64R- β_3 AR shows an enhanced cAMP response compared with WT- β_3 AR, when W64R- β_3 AR is coexpressed with AC-III.

MATERIALS AND METHODS

Materials

[¹²⁵I]Iodocyanopindolol ([¹²⁵I]ICYP) (2,200 Ci/mmol) and [³H]adenine (24.0–27.0 Ci/mmol) were obtained

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from Amersham Pharmacia Biotech (Arlington Heights, IL, USA). [^{14}C]cAMP (20.0–50.1 mCi/mmol) was obtained from Perkin Elmer Life Sciences (Boston, MA, USA). The mammalian expression vector pEF-BOS was a gift from Dr. S. Nagata (Osaka University, Osaka). The plasmids encoding for β_1 and γ_2 subunits of heterotrimeric G protein were kindly provided by Dr. S. Hoshino (University of Tokyo, Tokyo). The plasmids encoding the various AC isoforms were kindly provided by the following persons: AC-I, from Dr. R.T. Premont (Duke University Medical Center, Durham, NC, USA); AC-II and AC-III, from Dr. R.R. Reed (John Hopkins School of Medicine, Baltimore, MD, USA); and AC-VI, from Dr. Y. Ishikawa (Yokohama City University School of Medicine, Yokohama). CGP12177A, (–)isoproterenol · HCl and DEAE-dextran were from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was obtained from JRH Biosciences (Lenexa, KS, USA). Pfu DNA polymerase was from Stratagene (La Jolla, CA, USA). Sources of other reagents were mentioned in the previous report (9).

Construction and expression of WT- β_3 AR and mutant β_3 AR

Human WT- β_3 AR gene was cloned from HeLa genomic DNA by the polymerase chain reaction (PCR), and the second exon containing 6 amino acids at the carboxyl terminus was introduced by PCR as described (10). The mutation at position 64 (arginine instead of tryptophan) was introduced by PCR. The entire coding regions of WT- and W64R- β_3 ARs were inserted into expression vector pEF-BOS (11). These two β_3 AR constructs were used for transfection into COS-7 cells or JEG-3 cells by the DEAE-dextran method as described (12). Cells were plated onto a 100-mm dish for transfection. When β_3 AR was cotransfected with G_o or AC isoform, 1 μg of $G_{\alpha o}$ in pcDNA3 together with $G_{\beta 1}$ in pCMV5, $G_{\gamma 2}$ in pCMV5 and 5 μg of AC-I, -II, -III or -VI were used.

Radioligand binding assay

The crude membrane fraction from the transfected cells was prepared as described (9). Approximately 10 μg of membrane protein was used for radioligand binding studies, and 50 pM [^{125}I]ICYP was used for competition binding assays. Nonspecific binding was determined in the presence of 1 mM CGP12177A.

Determination of cAMP content in COS-7 and JEG-3 cells

The transfected COS-7 and JEG-3 cells were split into 6-well plates and incubated overnight (37°C, 5% CO_2) in culture medium containing 1 $\mu\text{Ci}/\text{ml}$ [^3H]adenine. The cells were stimulated in DMEM medium containing 20 mM HEPES (pH 7.4), 1 mM 3-isobutyl-1-methylxanthine and the various concentrations of agonists for 15 min at 37°C. The reaction was stopped, and generated [^3H]cAMP was iso-

lated as described (12). The cAMP production was calculated as the ratio of [^3H]cAMP to total [^3H]nucleotides per well (cAMP%/well) or normalized as fold stimulation, setting cAMP content without agonist as 1.0.

Data analyses

All data shown are mean values $\pm$ S.E.M. from three or four independent experiments. Equilibrium dissociation constants were determined from saturation isotherms, and competition curves were analyzed by a non-linear regression analysis using PRISM software (GraphPAD Software Inc., San Diego, CA, USA). The statistical significance was assessed with the *t* test or Dunnett's test.

RESULTS

Ligand binding characteristics

The affinities of various drugs for WT- or W64R- β_3 ARs were determined by ligand binding assay. The dissociation constant (K_d) of ICYP for W64R- β_3 AR was similar to the value of WT- β_3 AR (Table 1). Table 1 also shows that K_i values of the various agonists for W64R- β_3 AR are essentially the same as those for WT- β_3 AR. Thus, pharmacological characteristics of W64R- β_3 AR are similar to those of WT- β_3 AR.

Basal and receptor-stimulated cAMP responses

As CGP12177A is a partial agonist for β_3 AR and an antagonist for β_1 - and β_2 ARs, COS-7 cells did not respond to CGP12177A stimulation when the cells were not transfected with β_3 AR (Fig. 1). Stimulation by CGP12177A increased cAMP content in a dose-dependent manner in WT- and W64R- β_3 AR-expressing cells, and the EC_{50} value of CGP12177A to induce cAMP response of W64R- β_3 AR was essentially the same as that of WT- β_3 AR (Fig. 1). As the basal and agonist-stimulated cAMP accumulation is

Table 1. Ligands binding parameters of iodocyanopindolol, isoproterenol, norepinephrine, epinephrine and CGP12177A at the wild type and mutant β_3 -AR

	Wild type WT- β_3 -AR	Mutant W64R- β_3 -AR
K_d (nM)		
Iodocyanopindolol	40.3 $\pm$ 3.1	57.9 $\pm$ 2.3
K_i (nM)		
(–)Isoproterenol	270 $\pm$ 34	360 $\pm$ 53
(–)Norepinephrine	6000 $\pm$ 330	10000 $\pm$ 780
(–)Epinephrine	1400 $\pm$ 140	2600 $\pm$ 310
CGP12177A	19 $\pm$ 9	45 $\pm$ 14

[^{125}I]ICYP binding was determined on the membrane from COS-7 cells expressing the wild type or mutant β_3 -AR.

Fig. 1. The cAMP accumulation in COS-7 cells expressing wild type or mutant β_3 AR. cAMP accumulation dose-response curves for wild type (WT)- and mutant (Mut)-receptor transfected COS-7 cells, stimulated with CGP12177A (10 nM – 0.1 mM). Non: non-receptor-expressing COS-7 cells.

Fig. 2. Effect of receptor density on basal and CGP-stimulated adenylyl cyclase (AC) activity. The AC activity was measured in membranes derived from COS-7 cells transiently expressing WT (WT: solid circles, solid triangles)- or W64R (Mut: open circles, open triangles)- β_3 ARs at different receptor densities. The concentration of CGP12177A was 0.1 mM. The results are from 4 independent experiments assayed in duplicate. The solid lines indicate that the basal and CGP12177A-stimulated values for WT- β_3 AR, and the dashed lines indicate the basal and CGP12177A-stimulated cAMP contents for W64R- β_3 AR. Non: non-receptor-expressing COS-7 cells.

related to the expression level of the receptor, we determined the basal and CGP12177A-stimulated cAMP responses of WT- and W64R- β_3 ARs at the various expression levels. Figure 2 shows that the basal and CGP12177A-stimulated cAMP responses for two receptors are gradually increased by the increasing level of expression. The cAMP responses with or without CGP12177A were essentially the same between two β_3 ARs. This result indicates that W64R- β_3 AR activates G_s in similar potency and efficacy to WT- β_3 AR.

Fig. 3. Effect of pertussis toxin (PTX) on cAMP accumulation in WT- and W64R- β_3 ARs coexpressed with G_i protein in COS-7 cells. WT- and W64R (Mut)- β_3 ARs were stimulated with 0.1 mM CGP12177A. Non: non-receptor-expressing COS-7 cells.

Fig. 4. Effect of pertussis toxin (PTX) on cAMP accumulation in WT- and W64R- β_3 ARs coexpressed with G_o in COS-7 cells. WT- and W64R (Mut)- β_3 ARs were co-transfected with G_o in COS-7 cells, and these receptors were stimulated with 0.1 mM CGP12177A.

To examine the involvement of G_i and G_o in the CGP12177A-stimulated cAMP accumulation, we determined the role of G_i and G_o in the basal and CGP12177A-stimulated cAMP accumulation. To amplify the G_i - and G_o -mediated signal, we expressed β_3 AR together with G_i (expressed as $\alpha_{12} \cdot \beta_1 \cdot \gamma_2$) or G_o (expressed as $\alpha_o \cdot \beta_1 \cdot \gamma_2$). In G_i -over-expressing cells, the basal and CGP12177A-stimulated cAMP responses by W64R- β_3 AR stimulation were essentially the same as those by WT- β_3 AR stimulation. Pertussis toxin (PTX) treatment did not affect the basal and CGP12177A-stimulated cAMP responses (Fig. 3). When COS-7 cells were not transfected with β_3 AR, CGP12177A stimulation did not increase cAMP accumulation in the PTX-treated cells. This result indicates that PTX treatment itself does not affect the level or function of components involved in the β_3 AR/ G_s /AC system. The treatment of W64R- β_3 AR and G_i - but not G_o -over-expressing cells with PTX slightly but not significantly enhanced the cAMP response induced by CGP12177A stimulation (Fig. 4).

Fig. 5. Effect of co-transfection with AC isoforms on cAMP accumulation in WT- and W64R- β_3 ARs in JEG-3 cells. WT- and W64R (Mut)- β_3 ARs were co-transfected with AC-I, AC-II, AC-III or AC-VI in JEG-3 cells, and these receptors were then stimulated with 0.1 mM CGP12177A (A) or 0.1 mM isoproterenol (B). * $P < 0.05$

AC co-transfection

As neural stimulation of and cold exposure to brown adipose tissue selectively increases the expression of AC-III among AC-III, IV, V and VI (8), we examined the CGP12177A-induced cAMP response in cells expressing WT- or W64R- β_3 ARs under this particular condition. Therefore, WT- and W64R- β_3 ARs were co-expressed with AC-I, -II, -III or -VI in JEG-3 cells. As COS-7 but not JEG-3 cells express endogenous β AR, JEG-3 cells were used for stimulation by the full agonist isoproterenol. When W64R- β_3 AR was coexpressed with AC-III and AC-VI, CGP12177A stimulation enhanced the cAMP response as compared to WT- β_3 AR co-expressing with AC-III and AC-VI (Fig. 5A). When W64R- β_3 AR was stimulated by isoproterenol, the cAMP response was enhanced in AC-III-expressing cells (Fig. 5B). Co-transfection of AC-VI slightly but not significantly enhanced the isoproterenol-induced increase in cAMP accumulation. These results indicate that co-expression of W64R- β_3 AR with AC-III enhances the agonist-induced cAMP response as compared to co-expression of WT- β_3 AR with AC-III.

DISCUSSION

It has been reported that the mutation of tryptophan to

arginine at position 64 of human β_3 AR is associated with obesity, glucose intolerance, hypertension, dyslipidemia and early onset of non-insulin-dependent diabetes mellitus (2). However, two groups reported different results of biochemical characterization of mutated β_3 AR (W64R- β_3 AR). Pietri-Rouxel et al. reported the decreased ability of W64R- β_3 AR to maximally activate AC (7). In contrast to their report, Candelore et al. did not observe any significant differences between WT- and W64R- β_3 AR (6). In the present study, we compared the biochemical and pharmacological characteristics of W64R- β_3 AR to those of WT- β_3 AR. We used a transient assay system to avoid clonal variation of receptor-expressing cells. The binding experiments showed that the affinities of various agonists for W64R- β_3 AR were essentially the same as that of WT- β_3 AR. This result is consistent with those of the previous reports.

Basal and CGP12177A-stimulated cAMP responses of W64R- β_3 AR were almost the same as those of WT- β_3 AR. As the receptor-stimulated cAMP response is affected by the expression level of receptors, we determined the responses at various expression levels of the two β_3 ARs. We did not observe significant differences of basal and CGP12177A-induced cAMP responses between W64R- and WT- β_3 ARs. This result supports the conclusion of Candelore et al. (6), in which they did not detect any significant biochemical and pharmacological differences between the two receptors. Several groups reported that β_3 AR interacts not only with G_s but also with G_i (13–15) and found that β_3 AR activates extracellular receptor-regulated kinase in a G_i -dependent manner (13, 14). Gauthier et al. reported that G_i is involved in β_3 AR-mediated signaling in the heart (15). Therefore, we examined whether the functional contribution of G_i and G_o explains the decreased ability of W64R- β_3 AR to increase cAMP content. PTX treatment did not affect WT- and W64R- β_3 AR-induced cAMP responses even under the condition that G_i and G_o were over-expressed in a heterotrimeric form. This result suggests that coupling of two β_3 ARs with G_i and G_o did not contribute to the cAMP response in COS-7 cells. Although we could not observe any functional differences between the two β_3 ARs, we have found a difference between WT- and W64R- β_3 AR-mediated cAMP responses under a particular condition. Granneman demonstrated that rat brown adipose tissue expresses four kinds of AC isoforms, and the level of mRNA for AC-III is selectively increased by stimulation of the sympathetic nervous system (8). When AC-III was co-expressed with W64R- β_3 AR, CGP12177A- and isoproterenol-stimulated cAMP responses were significantly enhanced as compared to cAMP response in the cells expressing WT- β_3 AR and AC-III. However, the isoproterenol-stimulated cAMP response in W64R- β_3 AR co-expressing AC-VI was slightly but not significantly

enhanced, in contrast to co-expression with AC-III. CGP12177A is a partial agonist and isoproterenol is a full agonist. AC-VI but not AC-III is phosphorylated by protein kinase A, and the activity is impaired by the phosphorylation. Therefore, it is possible that isoproterenol stimulation slightly decreases AC-VI activity by protein kinase A activation. Although the expression levels of β_3 ARs and AC are important determinants for the receptor-mediated cAMP response, basal cAMP responses are essentially the same between WT- and W64R- β_3 ARs-expressing cells in AC-III and AC-VI expressing cells. Therefore, it is reasonable to conclude that the expression levels of β_3 ARs and AC are similar to each other, and the differential expression is not enough to explain the enhanced cAMP response induced by W64R- β_3 AR stimulation. Guan et al. demonstrated that third intracellular loop of β_3 AR is a critical region for coupling of β_3 AR with G_s (16). Because the mutation at position 64 is located at the first intracellular loop, this amino acid may directly participate in the interaction with G_s as previously suggested. As AC-III shows a modestly high turnover number of cAMP synthesis (17), the small increase in coupling efficiency of β_3 AR of W64R- β_3 AR to G_s may become apparent in the presence of AC-III. One of the consequences of the differential cAMP responses of WT- β_3 AR and W64R- β_3 AR is down-regulation of β_3 ARs. Because downregulation of β_3 AR is dependent on cAMP content (18), W64R- β_3 AR may down-regulate faster than WT- β_3 AR under the condition that AC-III is co-expressed.

In conclusion, the mutated β_3 AR at position 64 (W64R- β_3 AR) did not show any pharmacological and functional differences from WT- β_3 AR. However, W64R- β_3 AR showed the enhanced cAMP response when AC-III is co-expressed together with the receptor. It remains to be determined whether this difference plays a role in regulation of β_3 AR in vivo.

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